



Patent Application  
Docket No. UF-221C1XC1  
Serial No. 09/662,254

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner : Anne Marie S. Beckerleg, Ph.D.  
Art Unit : 1632  
Applicants : Richard W. Moyer, Yi Li, Allison L. Bawden  
Serial No. : 09/662,254  
Filed : September 14, 2000  
Confirm. No. : 2442  
For : Materials and Methods for Delivery and Expression of Heterologous DNA  
in Vertebrate Cells

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Assistant Commissioner for Patents  
Washington, D.C. 20231

DECLARATION OF RICHARD W. MOYER, Ph.D., UNDER 37 C.F.R. § 1.132

Sir:

I, Richard W. Moyer, Ph.D., of the University of Florida, hereby declare:  
THAT, I am an inventor on the above-referenced patent application;  
THAT, I have received the following degrees:

B.S. in Agricultural and Biological Chemistry with Minor in Chemistry;  
Pennsylvania State University; 1962

Ph.D. in Biochemistry with Minor in Organic Chemistry; University of California  
at Los Angeles; 1967;

THAT, I have been employed professionally as follows:

1987-present	Professor and Chairman, Department of Immunology and Medical Microbiology, College of Medicine, University of Florida
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1981-1987 Professor, Department of Microbiology, Vanderbilt University School of Medicine

1976-1981 Associate Professor, Department of Microbiology, Vanderbilt University School of Medicine

1970-1976 Assistant Professor of Biochemistry, Columbia University

1967-1969 Postdoctoral Fellow, Massachusetts Institute of Technology

6/1965-9/1965 Summer Trainee in Physiology, Woods Hole Marine Biology Laboratory

1963-1967 Research Assistant, Biochemistry, University of California at Los Angeles

1962-1963 Research Assistant, Biochemistry, University of Minnesota

THAT, I have published extensively in my field and some of the publications are as follows:

1. Bawden, A.L., K.J. Glassberg, J. Diggans, R. Shaw, W. Farmerie, R.W. Moyer (2000) "Complete genomic sequence of the *Ansacta moorei* entomopoxvirus: Analysis and comparison with other poxviruses" *Virology* 274(1):120-139;
2. Moon, K.B., P.C. Turner, R.W. Moyer (1999) "SPL-1-dependent host range of rabbitpox virus and complex formation with cathepsin G is associated with serpin motifs" *Journal of Virology* 73(11):8999-9010;
3. Turner, P.C., M.S. Sancho, S.R. Thoenes, A. Caputo, R.C. Bleackley, R.W. Moyer (1999) "Myxoma virus Serp2 is a weak inhibitor of granzyme B and interleukin-1beta-converting enzyme *in vitro* and unlike CrmA cannot block apoptosis in cowpox virus-infected cells" *Journal of Virology* 73(8):6394-6404;
4. Li, Y., S. Yuan, R.W. Moyer (1998) "The non-permissive infection of insect (gypsy moth) LD-652 cells by Vaccinia virus" *Virology* 248(1):74-82;

5. Li, Y. R.L. Hall, S.L. Yuan, R.W. Moyer (1998) "High-level expression of *Amsacta moorei* entomopoxvirus spheroidin depends on sequences within the gene" *J Gen Viro* 79(Pt 3):613-622;
6. Li, Y. R.L. Hall, R.W. Moyer (1997) "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *Journal of Virology* 71(12):9557-9562;
7. Peterson, B.O., R.L. Hall, R.W. Moyer, S. Shuman (1997) "Characterization of a DNA topoisomerase encoded by *Amsacta moorei* entomopoxvirus" *Virology* 230(2):197-206;
8. Graham, K.A., A.S. Lalani, J.L. Macen, T.L. Ness, M. Barry, L.Y. Liu, A. Lucas, I. Clark-Lewis, R.W. Moyer, G. McFadden (1997) "The T1/35kDa family of poxvirus-secreted proteins bind chemokines and modulate leukocyte influx into virus-infected tissues" *Virology* 229(1):12-24;
9. Winter, J., R.L. Hall, R.W. Moyer (1995) "The effect of inhibitors on the growth of the entomopoxvirus from *Amsacta moorei* in *Lymantria dispar* (gypsy moth) cells" *Virology* 211(2):462-473;
10. Turner, P.C., P.Y. Musy, R.W. Moyer (1994) *Viroceptors, Virokines, and Related Immune Modulators Encoded by DNA Viruses* (McFadden, G., ed.), R.G. Landes, Galveston, TX;
11. VanderLeek, M.L., J.A. Feller, G. Sorensen, W. Isaacson, C.L. Adams, D.J. Borde, N. Pfeiffer, T. Tran, R.W. Moyer, E.P.J. Gibbs (1994) "Evaluation of swinepox virus as a vaccine vector in pigs using an Aujeszky's disease (pseudorabies) virus gene insert coding for glycoproteins gp50 and gp63" *The Veterinary Record* 134:13-18;
12. Moyer, R.W. (1994) *Encyclopedia of Virology* (Webster, R.G., A. Granoff, eds.), Academic Press Ltd., London: pp. 392-397;
13. Ali, A.N., P.C. Turner, M.A. Brooks, R.W. Moyer (1994) "The SPI-1 gene of rabbitpox virus determines host range and is required for hemorrhagic pock formation" *Virology* 202:306-314;
14. Martinez Pomares, L., R.J. Stern, R.W. Moyer (1993) "The ps/hr gene (B5R open reading frame homolog) of rabbitpox virus controls pock color, is a component of extracellular enveloped virus, and is secreted into the medium" *Journal of Virology* 67:5450-5462;

15. Hall, R.L., R.W. Moyer (1993) "Identification of an *Amsacta* spheroidin-like protein within the occlusion bodies of *Choristoneura* entomopoxviruses" *Virology* 192:179-187
16. Bloom, D.C., R. Stern, M. Duke, D. Smith, R.W. Moyer (1993) "A revised *Hind*III map and sequence analysis of a large 'left-hand' non-essential region of the rabbit poxvirus genome" *Virus Res* 28:125-140;
17. Thompson, J.P., P.C. Turner, A.N. Ali, B.C. Crenshaw, R.W. Moyer (1993) "The effects of serpin gene mutations on the distinctive pathobiology of cowpox and rabbitpox virus following intranasal inoculation of Balb/c mice" *Virology* 197:328-338;
18. Massung, R.F., V. Jayarama, R.W. Moyer (1993) "DNA sequence analysis of conserved and unique regions of swinepox virus: Identification of genetic elements supporting phenotypic observations including a novel G protein-coupled receptor homologue" *Virology* 197:511-528;
19. Turner, P.C., R.W. Moyer (1992) "An orthopoxvirus serpinlike gene controls the ability of infected cells to fuse" *Journal of Virology* 66:2076-2085;
20. Turner, P.C., R.W. Moyer (1992) "A PCR-based method for manipulation of the vaccinia virus genome that eliminates the need for cloning" *Biotechniques* 13:764-771;
21. Massung, R.F., G. McFadden, R.W. Moyer (1992) "Nucleotide sequence of a unique near-terminal region of the tumorigenic poxvirus, Shope fibroma virus" *J Gen Virology* 73:2903-2911;
22. Gruidl, M.E., R.L. Hall, R.W. Moyer (1992) "Mapping and molecular characterization of a functional thymidine kinase from *Amsacta moorei* entomopoxvirus" *Virology* 186:507-516;
23. Hall, R.L., R.W. Moyer (1991) "Identification, cloning, and sequencing of a fragment of *Amsacta moorei* entomopoxvirus DNA containing the spheroidin gene and three vaccinia related ORFs" *Journal of Virology* 65:6516-6527;
24. Feller, Moyer *et al* (1991) "Isolation of molecular characterization of the swinepox virus thymidine kinase gene" *Virology* 183:578-585;
25. Brown, C.K., D.C. Bloom, R.W. Moyer (1991) "The nature of naturally occurring mutations in the hemagglutinin gene of vaccinia virus and the sequence of immediately adjacent genes" *Virus Genes* 5:235-242;

26. Brown, C.K., P.C. Turner, R.W. Moyer (1991) "Molecular characterization of the vaccinia hemagglutinin gene" *Journal of Virology* 65:3598-3606;
27. Bloom, D.C., K.M. Edwards, C. Hager, R.W. Moyer (1991) "Identification and characterization of two non-essential regions of the rabbitpox virus genome involved in virulence" *Journal of Virology* 65:1530-1542;
28. Massung, R.F., R.W. Moyer (1991) "The molecular biology of swinepox virus II. The infectious cycle" *Virology* 180:355-364;
29. Massung, R.F., R.W. Moyer (1991) "The molecular biology of swinepox virus I. Characterization of the viral DNA" *Virology* 180:347-354;
30. Turner, P.C., R.W. Moyer (1990) "The Molecular Pathogenesis of Poxviruses," in *Current Topics in Microbiology and Immunology*, P.C. Turner and R.W. Moyer, eds., Springer Verlag, New York, pp.125-153;
31. Turner, P.C., R.W. Moyer, eds. (1990) "The Molecular Pathogenesis of Poxviruses," in *The Poxviruses*, Springer Verlag, New York, pp.125-153;
32. Massung, R.F., R.W. Moyer (1989) "Orthopoxvirus gene expression in *Xenopus laevis* oocytes. I. A component of the virion is needed for late gene expression" *Journal of Virology* 64:2280-2289;
33. Turner, P.C., D.V. Young, J.B. Flanagan, R.W. Moyer (1989) "Interference with vaccinia virus growth caused by insertion of the coding sequences for poliovirus protease 2A" *Virology* 173:509-521;
34. Bloom, D.C., R. Massung, L. Savage, D.K. Morrison, R.W. Moyer (1989) "Recruitment to the cytoplasm of a cellular lamin-like protein from the nucleus during a poxvirus infection" *Virology* 169:115-126;
35. Edwards, K.M., T.C. Andrews, J. Van Savage, P. Palmer, R.W. Moyer (1988) "Poxvirus deletion mutants: Virulence and immunogenicity" *Microbial Pathogenesis* 4:325-333;
36. Moyer, R.W. (1987) "The role of the host cell nucleus in vaccinia virus morphogenesis" *Virus Research* 8:173-191;
37. Morrison, D.K., R.W. Moyer (1986) "Detection of a subunit of cellular PolII within highly purified preparations of RNA polymerase isolated from rabbit poxvirus virions" *Cell* 44:587-596;

38. Minnigan, H., R.W. Moyer (1985) "Intracellular location of rabbit poxvirus nucleic acid within infected cells as determined by *in situ* hybridization" *Journal of Virology* 55:634-643;
39. Brown, G.D., R.W. Moyer (1983) "The white pock mutants of rabbit poxvirus. V. *In vitro* translation of early host range mutant mRNA" *Virology* 126:381-390;
40. Moyer, R.W., R.L. Graves (1982) "The white pock mutants of rabbit poxvirus. IV. The late white pock ( $\mu$ ) host range (hr) mutants of rabbit poxvirus are blocked at the level of morphogenesis" *Virology* 119:332-346;
41. Moyer, R.W., R.L. Graves (1981) "The mechanism of cytoplasmic orthopoxvirus DNA replication" *Cell* 27:391-401;
42. Moyer, S.A., S.M. Horikami, R.W. Moyer (1981) "The effect of the host cell and heterologous viruses on VSV production," In *Replication of Negative Strand Viruses* (D.H.L. Bishop and R.W. Compans, eds.), Elsevier Press, New York, pp. 965-970;
43. Hamilton, D.H., R.W. Moyer, S.A. Moyer (1980) "Characterization of the non-permissive infection of rabbit cornea cells by vesicular stomatitis virus" *J Gen Virol* 49:273-287;
44. Moyer, R.W., R.L. Graves, C.T. Rothe (1980) "The white pock mutants of rabbit poxvirus. III. Terminal DNA sequence duplication and transposition in rabbit poxvirus" *Cell* 22:545-553;
45. Moyer, R.W., G.D. Brown, R.L. Graves (1980) "The white pock mutants of rabbit poxvirus. II. The early white pock ( $\mu$ ) host range (hr) mutants of rabbit poxvirus uncouple transcription and translation in non-permissive cells" *Virology* 106:234-249;
46. Moyer, R.W., C.T. Rothe (1980) "The white pock mutants of rabbit poxvirus. I. Spontaneous host range mutants contain deletions" *Virology* 102:119-132;
47. Herman, R.C., R.W. Moyer (1975) "In vivo repair of bacteriophage T5 DNA: An assay for viral growth control" *Virology* 66:393-407;
48. Moyer, R.W., A.S. Fu, J. Szabo (1972) "Regulation of bacteriophage development by Col I factors" *Journal of Virology* 9:804-812;

49. Moyer, R.W., R. Ramaley, P.D. Boyer (1967) "The formation and reactions of a nonphosphorylated high energy form of succinyl coenzyme A synthetase" *J Biol Chem* 242:4299; and
50. DeLuca, M., K.E. Ebner, D.E. Hultquist, G. Kreil, J.B. Peter, R.W. Moyer, P.D. Boyer (1963) "The isolation and identification of phosphohistidine from mitochondrial protein" *Biochem Z* 338:512;

THAT, I am an inventor on the following patents: U.S. Patent No. 5,935,777, "Entomopoxvirus Expression System," issued August 10, 1999; U.S. Patent No. 5,721,352, "Entomopoxvirus Expression System," issued February 24, 1998; U.S. Patent No. 5,651,972, "Use of Recombinant Swine Poxvirus as a Live Vaccine Vector," issued July 29, 1997; U.S. Patent No. 5,476,781, "Entomopoxvirus Spheroidin Gene Sequences," issued December 19, 1995; U.S. Patent 5,212,057, "Biological Systems for Constructing and Testing Viral Vaccines," issued May 18, 1993;

THAT, through my years of research, I have kept up to date on the technical literature and maintained contact with experts in the field by participating in professional meetings and seminars, and by direct personal contact. As a result, I am familiar with the general level of skill of those working in the fields of virology and molecular biology, and in particular the use of viral vectors in genetic engineering.

THAT, I have read and understood the specification and claims of the subject application and the Office Action dated September 4, 2001;

AND, being thus duly qualified, do further declare:

1. The entomopox virus vectors disclosed and claimed in this patent application have been found to be highly effective in delivering polynucleotides to vertebrate cells, resulting in the expression of genes encoded by the polynucleotides.
2. The patent Examiner states that at pages 13-14, bridging paragraph, of the patent application, it is indicated that entomopoxvirus cannot productively infect

mammalian cells and that gene expression is limited to early promoter activity. The patent Examiner also notes that the patent application indicates that late poxvirus promoters, such as AmEPV spheroidin or cowpox virus ATI, are inactive in mammalian cells infected with recombinant EPV. The patent Examiner then concludes "the skilled artisan would not predict that any and all promoter sequences could express a heterologous gene of interest when encoded by a recombinant entomopox virus."

3. The paragraph bridging pages 13-14 of the patent application refers to the general observation that when using entomopoxvirus vectors containing entomopox promoters, genes under the control of early entomopox promoters will be expressed in a vertebrate cell, but genes under the control of late entomopox promoters will not. The same paragraph of the patent application refers to two publications (Li *et al.* [1997] and Gauthier *et al.* [1995]), both of which describe experiments using entomopox virus vectors containing entomopox promoters. However, as explained below, the distinction between "early" promoters and "late" promoters only finds context with respect to poxvirus promoters (vertebrate and insect poxvirus promoters).
4. When insect poxviruses infect vertebrate cells, early and only early poxvirus promoters are active. This is likely because the early poxvirus transcription apparatus is packaged within the virion particle as part of creating virions from the previous infection. In vertebrate cells, following early promoter-driven expression, the infection then aborts and eventually the input virus particles disintegrate, after which the viral DNA is released into the vertebrate cell's cytoplasm; hence, the lack of late poxvirus promoter-driven expression in vertebrate cells. This observation is also made within the Li *et al.* publication (1997) at page 9557, second column, page 9560, second column, and page 9561, second column, which is cited by the patent Examiner. As indicated within the Li

*et al.* publication, at page 9561, second paragraph, and at page 71, lines 3-7, of the patent application, an exception to this phenomenon is when cells infected with EPV vectors containing late poxvirus promoters are supplied with certain additional factors *in trans* (e.g., by co-infection with vaccinia virus), which at least partially rescue late gene expression through activation of late promoters.

5. However, as taught within the patent application, early poxvirus promoters and (under certain circumstances) late poxvirus promoters are not the only promoters that can be used in an entomopoxvirus vector to achieve expression of a foreign gene within a vertebrate cell. Entomopoxvirus vectors containing genes under the control of non-poxvirus promoters can also be utilized, such as vertebrate host cell promoters and promoters from other viruses. This is documented at page 75, lines 11-24, and page 81, lines 15-24, of the patent application. Specifically, non-poxvirus promoters that are recognized by the vertebrate host cell's nuclear RNA polymerase, such as the cytomegalovirus (CMV) and herpes TK gene promoters, can be used following entomopoxvirus infection, entry of the viral DNA into the nucleus, and selection for stable transformation of the vertebrate host cell. Once transformed, those foreign genes that are under the control of the non-poxvirus promoters within the nuclear environment become activated, leading to expression of the foreign gene. Preferred promoters are those constitutive or regulatable promoters, such as the CMV or Herpes TK gene promoters, capable of promoting sufficient expression of the foreign DNA contained within the viral vector in a vertebrate cell.
6. The patent Examiner states that Example 11 of the patent application only examines expression of  $\beta$ -galactosidase expression at day two, following injection, "and does not correlate the level or duration of gene expression with any therapeutic effect." However, it would be expected that expression of the

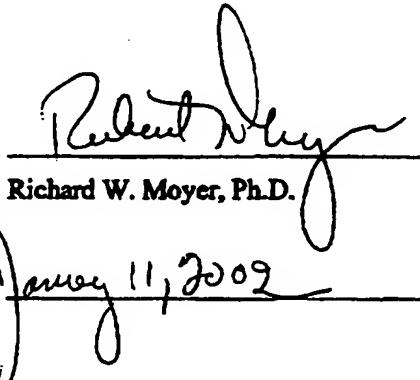
foreign gene,  $\beta$ -galactosidase in this case, would be sustained for a period beyond the two days at which time the tissue was excised.

7. The patent Examiner states the skilled artisan would not predict that the entomopox virus vectors of the invention could be used to express therapeutic levels of protein in lymphoid cells, which are associated with certain disorders, such as Burkitt's lymphoma. Although it has been reported in the literature that there is a greater level of expression in some cells (e.g., fibroblasts) than in other cells, such as lymphoid cells, one would expect that greater levels of expression could be achieved, for example, through the use of tissue-specific promoters and/or *in vitro* selection steps.
8. In summary, given the teachings of the patent application, one would expect that the entomopoxvirus vectors of the invention can be used to deliver and express foreign genes within a vertebrate cell, using a variety of promoters.

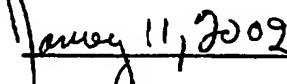
The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Signed:

  
Richard W. Moyer, Ph.D.

Date:

  
Jan 11, 2009



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1: Hum Gene Ther. 1997 Nov 20;8(17):2019-29.

Comment in:

- [Hum Gene Ther. 1997 Nov 20;8\(17\):2009-10.](#)

**Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression.**

**Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS.**

Department of Surgery, University of Michigan, Ann Arbor 48109-0331, USA.

One of the major limitations to current gene therapy is the low-level and transient vector gene expression due to poorly defined mechanisms, possibly including promoter attenuation or extinction. Because the application of gene therapy vectors *in vivo* induces cytokine production through specific or nonspecific immune responses, we hypothesized that cytokine-mediated signals may alter vector gene expression. Our data indicate that the cytokines interferon-gamma (IFN-gamma) and tumor necrosis factor-alpha (TNF-alpha) inhibit transgene expression from certain widely used viral promoters/enhancers (cytomegalovirus, Rous sarcoma virus, simian virus 40, Moloney murine leukemia virus long terminal repeat) delivered by adenoviral, retroviral or plasmid vectors *in vitro*. A constitutive cellular promoter (beta-actin) is less sensitive to these cytokine effects. Inhibition is at the mRNA level and cytokines do not cause vector DNA degradation, inhibit total cellular protein synthesis, or kill infected/transfected cells. Administration of neutralizing anti-IFN-gamma monoclonal antibody results in enhanced transgene expression *in vivo*. Thus, standard gene therapy vectors in current use may be improved by altering cytokine-responsive regulatory elements. Determination of the mechanisms involved in cytokine-regulated vector gene expression may improve the understanding of the cellular disposition of vectors for gene transfer and gene therapy.

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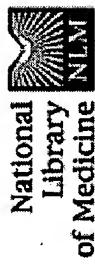
UF HSC Libraries  
Gainesville In Print**Transient immunosuppression allows transgene expression following readministration of adeno-associated viral vectors.****Manning WC, Zhou S, Bland MP, Escobedo JA, Dwarki V.**

Chiron Corporation, Emeryville, CA 94608, USA.

Adeno-associated viral (AAV) vectors have much promise in gene therapy. Among the many properties that make AAV an ideal vector for gene therapy are its ability to infect both dividing and nondividing cells and the longevity of expression in tissues such as brain, skeletal muscle, and liver. However, like other viral vectors, readministration of vector is limited because of the host's immune response to viral components of the vector.

Using class I, class II, and CD40 ligand (CD40L)-deficient mice, we demonstrate that neutralizing antibodies to the viral capsid proteins prevent transgene expression following readministration of rAAV vectors. Transient immunosuppression of mice by treatment with antibody to CD4 at the time of primary infection allowed transgene expression after readministration of rAAV vectors to animals. Transient immunosuppression with antibody to CD40L had only a modest effect on the efficacy of readministration. The ability to readminister virus was inversely correlated with both AAV capsid enzyme-linked immunosorbent assay titers and AAV neutralizing antibody titers. These studies demonstrate that readministration of rAAV can be accomplished by down regulating the anti-AAV immune response and suggest the use of repeated administration of rAAV as a viable form of therapy for the treatment of chronic diseases.

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## Constitutive Expression of phVEGF<sub>165</sub> After Intramuscular Gene Transfer Promotes Collateral Vessel Development in Patients With Critical Limb Ischemia

Iris Baumgartner, MD; Ann Pieczek, RN; Orit Manor, PhD; Richard Blair, MD; Marianne Kearney, BS; Kenneth Walsh, PhD; Jeffrey M. Isner, MD

**Background**—Preclinical studies have indicated that angiogenic growth factors can stimulate the development of collateral arteries, a concept called “therapeutic angiogenesis.” The objectives of this phase 1 clinical trial were (1) to document the safety and feasibility of intramuscular gene transfer by use of naked plasmid DNA encoding an endothelial cell mitogen and (2) to analyze potential therapeutic benefits in patients with critical limb ischemia.

**Methods and Results**—Gene transfer was performed in 10 limbs of 9 patients with nonhealing ischemic ulcers (n=7/10) and/or rest pain (n=10/10) due to peripheral arterial disease. A total dose of 4000 µg of naked plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor (phVEGF<sub>165</sub>) was injected directly into the muscles of the ischemic limb. Gene expression was documented by a transient increase in serum levels of VEGF monitored by ELISA. The ankle-brachial index improved significantly (0.33±0.05 to 0.48±0.03,  $P=.02$ ); newly visible collateral blood vessels were directly documented by contrast angiography in 7 limbs; and magnetic resonance angiography showed qualitative evidence of improved distal flow in 8 limbs. Ischemic ulcers healed or markedly improved in 4 of 7 limbs, including successful limb salvage in 3 patients recommended for below-knee amputation. Tissue specimens obtained from an amputee 10 weeks after gene therapy showed foci of proliferating endothelial cells by immunohistochemistry. PCR and Southern blot analyses indicated persistence of small amounts of plasmid DNA. Complications were limited to transient lower-extremity edema in 6 patients, consistent with VEGF enhancement of vascular permeability.

**Conclusions**—These findings may be cautiously interpreted to indicate that intramuscular injection of naked plasmid DNA achieves constitutive overexpression of VEGF sufficient to induce therapeutic angiogenesis in selected patients with critical limb ischemia. (*Circulation*. 1998;97:1114-1123.)

**Key Words:** angiogenesis ■ genes ■ ischemia ■ growth substances

Critical limb ischemia is estimated to develop in ≈500 to 1000 individuals per million per year.<sup>1</sup> In a large proportion of these patients, the anatomic extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization, and the disease thus frequently follows an inexorable downhill course.<sup>2,3</sup> Psychological testing of such patients has disclosed quality-of-life indices similar to those of patients with cancer in the terminal phase of their illness.<sup>4</sup> As concluded in the Consensus Document of the European Working Group on Critical Limb Ischemia,<sup>1</sup> no pharmacological treatment has been shown to favorably affect the natural history of critical limb ischemia.<sup>5</sup> Indeed, amputation, despite its associated morbidity, mortality, and functional implications,<sup>1,6-8</sup> is often recommended as a solution to the disabling symptoms, in particular excruciating ischemic rest pain, of critical limb ischemia.<sup>9-12</sup> A second major amputation

will be required in nearly 10% of such patients. Despite the use of prosthetics and rehabilitation, reestablishment of full mobility is inconsistently achieved, particularly in the elderly. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling.

### See p 1108

Preclinical studies have indicated that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral<sup>13,14</sup> and myocardial<sup>15-17</sup> ischemia, a concept called therapeutic angiogenesis. Several of these studies have used VEGF, also known as vascular permeability factor, a secreted endothelial-cell mitogen with high-affinity binding sites limited to endothelial cells.<sup>18-22</sup> Endothelial cell specificity has been considered to represent an important advantage of VEGF for therapeutic angiogenesis, because endothelial cells represent the critical cellular element responsible for new vessel formation.<sup>23-25</sup>

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**Selected Abbreviations and Acronyms**

ABI	ankle-brachial index
MRA	magnetic resonance angiography
PCR	polymerase chain reaction
phVEGF <sub>165</sub>	plasmid encoding 165-amino-acid isoform of human VEGF
TBI	toe-brachial index
VEGF	vascular endothelial growth factor

We recently demonstrated angiographic and histological evidence of angiogenesis after intra-arterial gene transfer of naked plasmid DNA encoding human VEGF in a patient with critical limb ischemia.<sup>26</sup> In this report, we present the results of intramuscular phVEGF<sub>165</sub> gene transfer performed in an initial phase 1 clinical trial comprising 9 patients with 10 critically ischemic limbs.

**Methods****Patients**

Patients qualified for intramuscular gene therapy if they (1) had chronic critical limb ischemia<sup>1</sup> including rest pain and/or nonhealing ischemic ulcers present for a minimum of 4 weeks without evidence of improvement in response to conventional therapies and (2) were not optimal candidates for surgical or percutaneous revascularization.<sup>27</sup> Requisite hemodynamic deficit included a resting ABI <0.6 and/or TBI <0.3 in the affected limb on 2 consecutive examinations performed at least 1 week apart. Criteria used to describe a change in limb status were adapted from standards recommended by the Society for Vascular Surgery/North American Chapter and International Society for Cardiovascular Surgery.<sup>3,28</sup> Patients were allowed to continue on aspirin and coumarin, provided that these therapies had been used for a minimum of 6 months before gene transfer. Vasoactive medications were discontinued unless prescribed for cardiac disease or systemic hypertension. All patients gave written informed consent for their participation. The study was designed as a phase 1, nonrandomized study to document the safety of intramuscular phVEGF<sub>165</sub> gene transfer and to monitor patients as well for evidence of bioactivity. This study design was unanimously approved by the Recombinant DNA Advisory Committee of the National Institutes of Health, by the Human Institutional Review Board and Institutional Biosafety Committee of St Elizabeth's Medical Center, and by the US Food and Drug Administration.

**Plasmid DNA (phVEGF<sub>165</sub>)**

All patients received a eukaryotic expression vector encoding the VEGF<sub>165</sub> gene<sup>29</sup> transcriptionally regulated by the cytomegalovirus promoter/enhancer.<sup>26</sup> Preparation and purification of the plasmid from cultures of phVEGF<sub>165</sub>-transformed *Escherichia coli* were performed in the Human Gene Therapy Laboratory at St Elizabeth's Medical Center by the column method (Qiagen Mega Kit, Qiagen, Inc). The purified plasmid was stored in vials and pooled for quality control analyses.

**Intramuscular phVEGF<sub>165</sub> Transfer**

Aliquots of 500 µg of VEGF<sub>165</sub> pDNA were diluted in sterile saline, and 4 aliquots (total, 2000 µg) were administered into calf and/or distal thigh muscles of the patients by direct intramuscular injection into the ischemic limb. The injection sites were arbitrarily selected according to available muscle mass and included sites above as well as below the knee. The volume of each of the 4 injectates per limb was progressively increased during the course of the study from 0.75 mL (3 treatments) to 3 mL (6 treatments) to 5 mL (11 treatments). Four weeks after the first 2000-µg injection, a second 2000-µg injection was administered, increasing the total amount of pDNA to 4000 µg per patient. One patient was treated for bilateral critical

limb ischemia with a total amount of 8000 µg pDNA (4000 µg per limb).

**Serum VEGF Levels**

ELISAs were performed at baseline and weekly up to 12 weeks after the initial treatment of 7 limbs to detect evidence of gene expression at the protein level. Samples were immediately centrifuged for 20 minutes at 3600 rpm at 4°C, and the serum was stored at -20°C until analysis. Serum VEGF was determined with an immunoassay according to the manufacturer's instructions (R&D Systems). Results were compared with a standard curve of human VEGF with a lower detection limit of 5 pg/mL. Samples were checked by serial dilution and were performed at least in duplicate.

**Hemodynamic and Angiographic Assessment**

Patients were followed up on a weekly basis within the first 8 weeks after gene therapy and at monthly intervals thereafter. Ischemic ulcers were documented by color photography. Resting ABI and TBI were calculated by the quotient of absolute ankle or toe pressure to brachial pressure.<sup>30</sup> Intra-arterial digital subtraction angiography and MRA were performed within 1 week before and 4 weeks after each treatment and 3 months after the latter of 2 intramuscular injections. Digital subtraction angiography was performed as a selective single-leg runoff study using undiluted nonionic contrast media (Isovue-370, Squibb Diagnostics). A minimum of 2 images (early and late frames) at the thigh, knee, calf, and ankle/foot levels were recorded by digital acquisition and hard copies in a 35×45-cm format. The diameter of newly visible collateral vessels was assessed by comparison with a 0.09-in-diameter reference wire taped to the skin. MRA was performed with a 1.0-T superconducting system (Impact, Siemens) by means of a transmit-receive extremity coil, a body coil, or both and commercially available pulse sequences. A multisector two-dimensional time-of-flight gradient echo sequence without intravenous contrast medium was used.<sup>31</sup> All axial images were reconstructed by use of the maximum-pixel-intensity algorithm at intervals of 60°.

**Immunohistochemistry**

Double immunohistochemical staining for proliferating endothelial cells was performed as previously described.<sup>32</sup> Bound antibody was then detected with an alkaline phosphatase kit (Biogenex Laboratories). Complexes were visualized with fast red substrate (Biogenex Laboratories). A counterstain of 10% Gill hematoxylin was applied before coverslips were applied.

**DNA Analysis**

Skin specimens at the site of gene injection and muscle specimens near or remote from the site of gene injection were retrieved from 2 amputees 8 and 10 weeks after intramuscular phVEGF<sub>165</sub> transfer, respectively (patients 4 and 10, Table). Tissue was processed with a genomic DNA isolation kit (A.S.A.P., Boehringer Mannheim). For PCR analysis, primer sets unique to the promoter and VEGF coding region of phVEGF<sub>165</sub> were selected. For Southern analysis, EcoRI-digested total cellular DNA (30 µg) and purified phVEGF<sub>165</sub> DNA (0.5 µg) were subjected to 0.8% agarose electrophoresis. The predicted sizes of EcoRI-digested plasmid fragments were 998 and 4703 bp. DNA blotted to a nylon membrane (Amersham, Life Science) was hybridized with two <sup>32</sup>P-labeled phVEGF<sub>165</sub>-specific probes (*ncol*-digested 679-bp phVEGF<sub>165</sub> fragment, position 389 to 1068; *aval*-digested 787-bp phVEGF<sub>165</sub> fragment, position 991 to 1778), washed, and exposed to Hyperfilm MP (Amersham, Life Science).

**Statistical Analysis**

Data are reported as mean±SEM. Comparisons between paired variables were performed with the nonparametric Friedman test and Wilcoxon rank sum test. All statistical tests were two-tailed, with a significance level of *P*<.05.

Clinical, Hemodynamic, Angiographic, Laboratory, and Molecular Findings Before and After Intramuscular phVEGF<sub>165</sub> Gene Transfer

No.	Sex	Age, y	Clinical History and Findings Before Gene Therapy				Outcome After Gene Transfer	
			Cigs, pk/y	DM	Previous Treatment	Signs/Symptoms	Limb Status	DSA Findings
1	F	33†	30	0	4 bypass grafts, 3 rev., prostaglandins	Calf ulcer, toe gangrene (digit I)	ABI +0.24; complete healing→limb salvage	New collaterals, 200–400 μm
2	F	53	0	+	3 bypass grafts, 1 PTA, prostaglandins	Toe gangrene (digit V)	ABI +0.12; complete healing	New collaterals, 200–400 μm
3	M	77	0	+	None	Toe gangrene (digits I, IV)	TBI +0.11; gangrene/osteomyelitis→BKA	New collaterals, 200–400 μm
4	F	39†	20	0	Sympathectomy	Forefoot gangrene	ABI +0.27; forefoot necrosis→BKA	New collaterals, 200–400 μm
5	M	74	90	0	1 PTA	Rest pain	ABI +0.15; rest pain resolved	New collaterals, 200–800 μm
6	F	84	40	0	6 bypass grafts, 1 PTA	Toe gangrene (digits I–V)	ABI +0.22; toe amputation→limb salvage	None
7	F	80	20	0	1 bypass graft	Rest pain	ABI unchanged, rest pain resolved	New collaterals, 200–800 μm
8*	F	39	20	0	Sympathectomy	Heel ulcer, toe gangrene (digits I–IV)	ABI +0.22; toe amputation→limb salvage	New collaterals, 200–>800 μm
9	M	54	30	0	4 bypass grafts, 2 rev., 1 PTA	Rest pain	TBI +0.18; rest pain resolved	None
10	M	54	70	0	6 bypass grafts, 1 PTA	Toe gangrene (digits I, III, IV)	No change in ABI/TBI, BKA	None

No. indicates consecutively treated ischemic limbs; Cigs, current cigarette smoker; pk/y, pack years of cigarette smoking; DM, diabetes mellitus (non-insulin-dependent DM, oral medication); DSA, digital subtraction angiography; BI, baseline; rev., surgical revisions; PTA, percutaneous transluminal angioplasty; TBI (ABI incompressible); BKA, below-knee amputation; ND, not done; and pos., positive.

\*No. 8 and 4 identical patient (bilateral treatment).

†Suspected Buerger's disease (stopped smoking >3 months before study entry).

## Results

Demographic and clinical data for the 5 women and 4 men (mean age, 59±19 years) treated with phVEGF<sub>165</sub> are shown in the Table. Average length of follow-up at the time of this report was 6±3 months (range, 2 to 11 months). Local intramuscular gene transfer induced no or mild local discomfort up to 72 hours after the injection. Serial creatine phosphokinase measurements remained in the normal range, there were no signs of systemic or local inflammatory reactions, and no patient developed antibodies to VEGF. To date, neither loss of visual acuity nor change in funduscopic examination due to diabetic retinopathy<sup>33</sup> has been observed in any patient treated with phVEGF<sub>165</sub> gene transfer. Likewise, no development of a latent neoplasm<sup>34</sup> has been observed. The only complication seen was transient lower-extremity edema, consistent with VEGF enhancement of vascular permeability.<sup>35</sup>

## Transgene Expression

Blood levels of VEGF transiently peaked 1 to 3 weeks after gene transfer in 7 patients in whom weekly blood samples were obtained (Fig 1). (In 2 patients, baseline and/or more than two follow-up blood samples were not obtained.) Indirect clinical evidence of VEGF overexpression was evident from the development of peripheral edema (+1 to +4 by

gross inspection) in the 6 patients with ischemic ulcers. In 4 of these patients, edema was limited to the treated limb, whereas in 2 patients, the contralateral limb was affected as well, albeit less severely. The onset of edema corresponded temporally to the rise in serum VEGF levels.

## Noninvasive Arterial Testing

Absolute systolic ankle or toe pressure increased in 8 limbs after gene transfer and was unchanged in 1 limb at the time of the most recent follow-up (53±4.8 at baseline, 66±4.6 most recent follow-up,  $P=.008$ ). ABI and/or TBI increased from 0.33±0.05 (range, 0 to 0.58; n=10) at baseline to 0.43±0.04 (0.22 to 0.57,  $P=.028$ ; n=10) at 4 weeks, to 0.45±0.04 (0.27 to 0.59,  $P=.016$ ; n=10) at 8 weeks, and to 0.48±0.03 (0.27 to 0.67,  $P=.017$ ; n=8) at 12 weeks (Fig 2). Improvement in the pressure index was sustained, but the increases in values obtained after the second (4-week) injection were not significantly different from measurements made 4 weeks after the initial injection. Exercise performance improved in all 5 patients with rest pain or ischemic ulcers who were able to perform graded treadmill exercise.<sup>36</sup> All patients experienced a significant increase in pain-free walking time (2.5±1.1 minutes before gene therapy versus 3.8±1.5 minutes at an average of 13 weeks after gene therapy,  $P=.043$ ). A statistically significant increase in absolute, claudication-limited

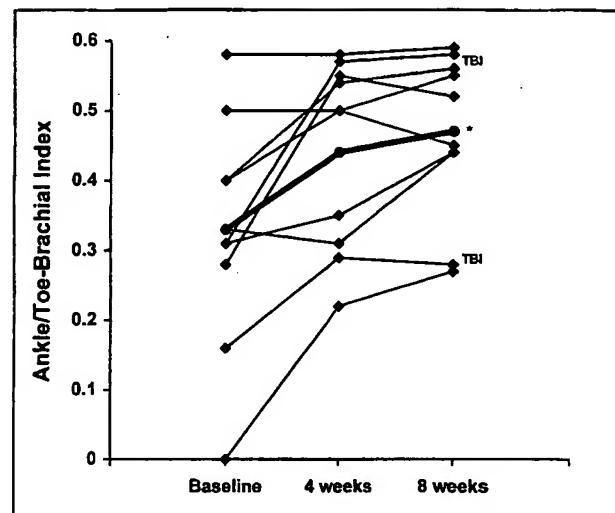
Continued

BI	VEGF Level, pg/mL		Molecular Findings
	1st	2nd	
47	223	607	
36	ND	ND	
46	131	780	
30	59	888	PCR pos. in skin + muscle specimens; Southern pos. in muscle specimens
62	300	96	
29	164	80	
40	44	223	
ND	ND	ND	
0	113	83	
ND	ND	ND	PCR pos. in skin + muscle specimens; Southern pos. in muscle specimens

walking time ( $4.2 \pm 2.1$  minutes before versus  $6.7 \pm 2.9$  minutes after gene therapy,  $P=.018$ ) was documented as well. Two patients reached the target end point of 10 minutes of exercise.

### Angiography

Digital subtraction angiography showed newly visible collateral vessels at the knee, calf, and ankle levels in 7 of 10 treated ischemic limbs. The luminal diameter of the newly visible vessels ranged from 200 to  $>800 \mu\text{m}$ , although most were closer to  $200 \mu\text{m}$ ; the latter frequently appeared as a "blush" of innumerable collaterals (Fig 3A and 3B). Fol-

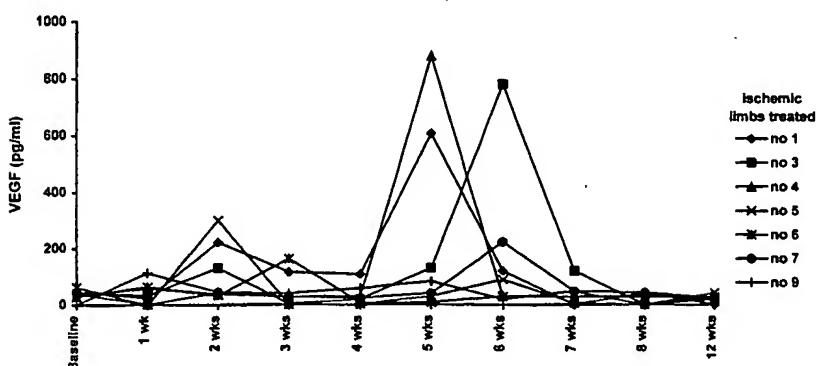


**Figure 2.** Gain in ABI and/or TBI in 10 limbs 4 and 8 weeks after intramuscular phVEGF<sub>165</sub> gene transfer. \*Mean values,  $P=.02$ .

low-up angiograms disclosed no evidence of collateral artery regression in any patients. Serial magnetic resonance angiograms of the ischemic limb disclosed qualitative evidence of improved distal blood flow in 8 limbs, including enhancement of signal intensity in previously identified vessels, and an increase in the number of newly visible collaterals (Fig 3C and 3D).

### Change in Limb Status and Ischemic Rest Pain

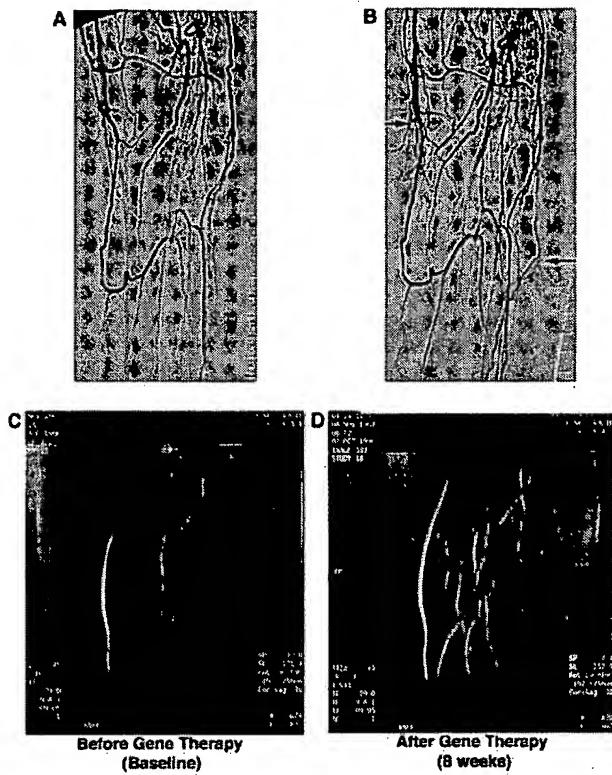
Therapeutic benefit was demonstrated by regression of rest pain and/or improved tissue integrity in the ischemic limb. Limb salvage, for example, was achieved in a 33-year-old woman (patient 1, Table), who had undergone 7 unsuccessful surgical reconstructions at another hospital. She presented with a necrotic great toe and a  $9 \times 3$ -cm ischemic ulcer at the site of vein harvest in her distal left limb (Fig 4). The ulcer had failed to respond to 6 months of conservative measures, during the last 3 of which she had been treated with methadone, oxycodone/acetaminophen, amitriptyline hydrochloride, and a fentanyl patch. She had been advised by her vascular surgeons to undergo below-knee amputation. Within 8 weeks after gene transfer, her ABI had increased by 0.24, and the ulcer dimensions had diminished sufficiently to permit placement of a split-thickness skin graft. The graft healed successfully and remained healed at 9-month fol-



Ischemic limbs treated

- no 1
- no 3
- ▲— no 4
- ★— no 5
- ◆— no 6
- no 7
- no 9

**Figure 1.** Serial levels of VEGF determined by ELISA disclosed a transient elevation 1 to 2 weeks after intramuscular (phVEGF<sub>165</sub>) gene transfer. Baseline and/or weekly follow-up venous blood samples, which were incomplete in 3 of 10 treated limbs (patients 2, 8, and 10 in the Table), are not shown.



**Figure 3.** A and B, Newly visible collateral vessels at calf level 8 weeks after phVEGF<sub>165</sub> gene transfer. Luminal diameter of newly visible vessels ranged from 200 to >800  $\mu$ m (arrow); most were closer to 200  $\mu$ m, and these frequently appeared as a blush of innumerable collaterals. C and D, MRA before and 8 weeks after gene therapy. After gene therapy, signal enhancement is clearly evident, consistent with improved flow in ischemic limb.

low-up (Fig 4). A second patient, a 39-year-old woman (patients 4 and 8, Table), presented with a 3-month history of gangrene of the distal half of her right foot. Although the ABI in her right limb improved by 0.27 after gene transfer, the forefoot gangrene was not reversed, and she underwent right below-knee amputation. While she was being treated for the right limb, however, she developed gangrene in the left limb (Fig 5). After gene transfer to the left limb, the ABI in her left lower extremity increased by 0.22 in association with angiographically demonstrable, newly visible collateral vessels. Although she required amputation of the left great toe, the operative site healed promptly, and her remaining 4 toes and heel recovered completely, including restoration of normal nail growth (Fig 5). In 2 other patients, complete (patient 2, Table) or partial (patient 6, Table) healing of ischemic ulcers present for 12 and 2 months, respectively, avoided major limb amputation. In the 3 patients (patients 5, 7, and 9, Table) who presented with rest pain (of 6, 5, and 3 months' duration, respectively) unassociated with loss of tissue integrity, rest pain resolved completely in all 3 patients after gene transfer and has remained so at the most recent follow-up.

For the total group of 10 limbs, frequency of ischemic rest pain expressed as afflicted nights per week decreased significantly ( $5.9 \pm 2.1$  at baseline versus  $1.5 \pm 2.8$  at 8-week follow-up,  $P=.043$ ). On the basis of criteria proposed by Rutherford et al,<sup>3</sup> limb status improved in 9 of 10 extremities treated (Table). Moderate improvement, including both an

upward shift in clinical category ( $\geq 1$  clinical category in patients with rest pain and  $\geq 2$  categories in patients with tissue loss) and an increase of  $>0.1$  in the ABI, was documented in 5 patients.

### Immunohistochemistry and Molecular Analyses

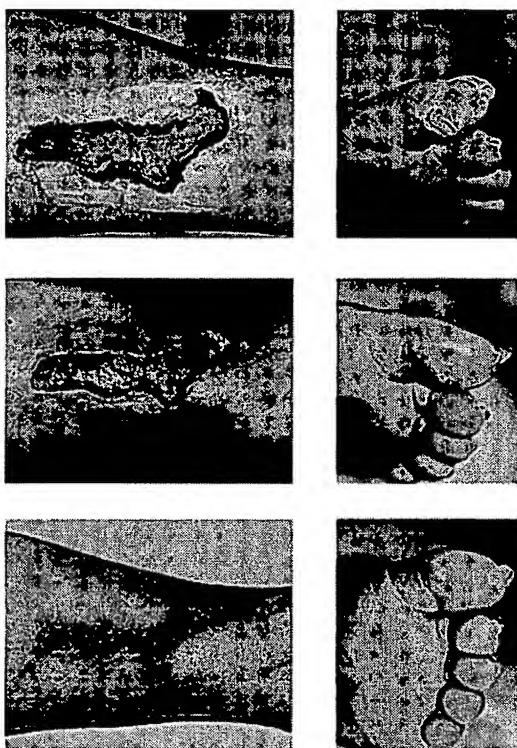
Tissue specimens retrieved from 1 amputee 10 weeks after gene therapy showed foci of proliferating endothelial cells (Fig 6A). This finding was particularly striking given that endothelial cell proliferation is nearly absent in normal arteries.<sup>37</sup> PCR performed on these samples indicated persistence and anatomic redistribution of DNA fragments unique to phVEGF<sub>165</sub>. Noteworthy amplification of DNA fragments was shown in muscle and skin samples derived from the site of injection as well as in several muscle samples remote from the injection site (Fig 6B). Southern blot analysis confirmed persistence of intact plasmid DNA in muscle specimens derived from 2 amputees 8 and 10 weeks after gene therapy (patients 4 and 10, Table) (Fig 6C).

### Discussion

The natural history of critical limb ischemia has been well documented to have an inexorable downhill course.<sup>3</sup> The inclusion criteria for this study were drafted to restrict treatment to patients in whom the natural history of critical limb ischemia had been established previously. Seven of the 10 limbs had developed frank gangrene. Although inclusion criteria required a minimum of 4 weeks of conservative measures without evidence of improvement, in reality, signs and/or symptoms of critical limb ischemia had been present in all cases for 2 to 12 months before gene therapy. Among this series of 9 patients (10 limbs), 6 developed critical limb ischemia despite having undergone as many as 7 vascular surgical reconstructions. Seven patients had been specifically advised to undergo limb amputation. All were using analgesic, typically  $\geq 1$  narcotic, medications. Spontaneous resolution of rest pain and/or healing of an ischemic ulcer in patients like these with critical limb ischemia has not to our knowledge been reported previously.<sup>1</sup> Furthermore, because VEGF had not been administered previously as recombinant protein, no data were available from any source to indicate either the safety or bioactivity of any dose of phVEGF<sub>165</sub>. Accordingly, the design of this phase 1 trial, unanimously approved by the Recombinant DNA Advisory Committee and the US Food and Drug Administration, was conducted as a nonrandomized, consecutive treatment series, similar to phase 1 oncology protocols used to study new chemotherapeutic agents administered to human subjects.

Analysis of gene expression at the protein level by use of an ELISA assay for VEGF documented a transient peak of the gene product in the systemic circulation 1 to 3 weeks after gene transfer in 7 patients. Further evidence of gene expression was observed in 6 patients, who developed temporally related peripheral edema, including 2 with bilateral edema. Parenthetically, the latter finding constitutes what is to the best of our knowledge the first demonstration that VEGF may augment vascular permeability in human subjects.

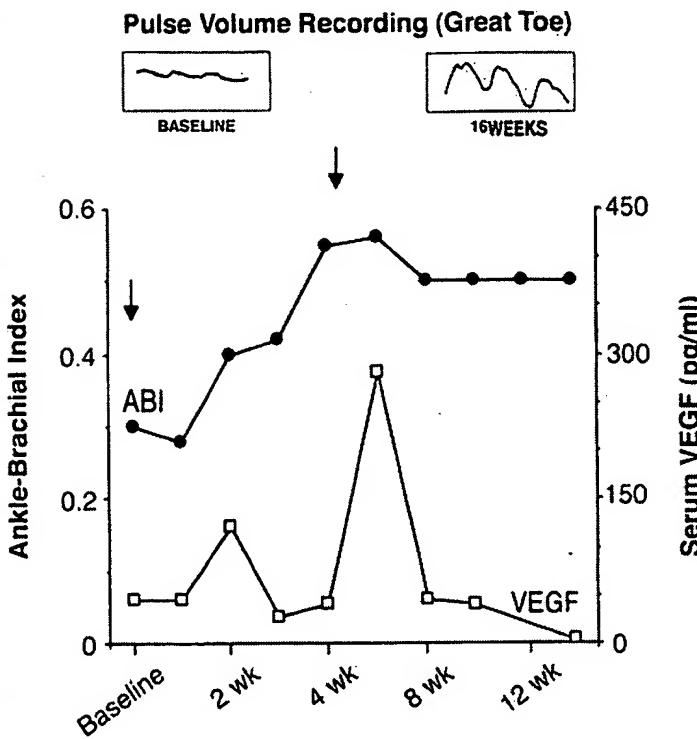
In most patients, treatment was sufficient to achieve clinically significant modulation of the recipient phenotype.



**Figure 4.** Limb salvage after gene therapy in a 33-year-old woman (patient 1, Table). Left top, Nonhealing wound on medial aspect of calf and ischemic necrosis involving great toe. Left middle, Ingrowth of granulation tissue in calf wound, healing of great toe. Left bottom, Three months after gene transfer, healing of split-thickness skin graft at wound site and full resolution of great toe necrosis. Before gene therapy, patient was wheelchair-bound on multiple analgesics, including methadone, amitriptyline hydrochloride, clonidine, oxycodone/acetaminophen, and a fentanyl patch. Three months after gene transfer, she was freely ambulatory and had been successfully weaned from all analgesics. Right, Evidence of phVEGF<sub>165</sub> bioavailability documented by an increase in ABI. The ABI progressively increased from 0.28 before to 0.56 after gene therapy (weeks refer to time after transfection). This was associated with development of a phasic pulse volume recording compared with nonphasic tracing recorded at baseline. Vertical arrows indicate timing of each of the 2 intramuscular phVEGF<sub>165</sub> injections.

Noninvasive studies documented hemodynamic evidence of improved limb perfusion that satisfies outcome criteria proposed to assess the results of surgical reconstruction or percutaneous revascularization.<sup>28</sup> Absolute ankle and/or toe pressure increased in 8 limbs after gene therapy ( $P=.008$ ). ABI and/or TBI increased from  $0.33 \pm 0.05$  at baseline to  $0.48 \pm 0.03$  at 12 weeks ( $P=.017$ ). To put this in perspective, an increase of  $>0.1$  in the ABI is considered indicative of a successful surgical or percutaneous intervention.<sup>28</sup> To the best of our knowledge, such improvement has not previously been achieved spontaneously or with medical therapy in patients with critical limb ischemia.

Similarly, angiographic demonstration of newly visible collateral vessels, accompanied here by noninvasive (MRA) evidence of improved blood flow, has to the best of our knowledge not been reported previously in response to any therapeutic intervention. Indeed, previous reports have indicated that current methods used to perform diagnostic contrast angiography cannot provide images of arteries measuring  $<200 \mu\text{m}$  in diameter<sup>18</sup>; the spatial resolution of images obtained by MRA is even less. Using synchrotron radiation microangiography to assess collateral artery development after VEGF gene transfer in a rat model of hindlimb ischemia, Takeshita et al<sup>18</sup> showed that neovascularization included a substantial contribution of vessels  $<180 \mu\text{m}$  in diameter. Thus, conventional angiographic techniques used in the

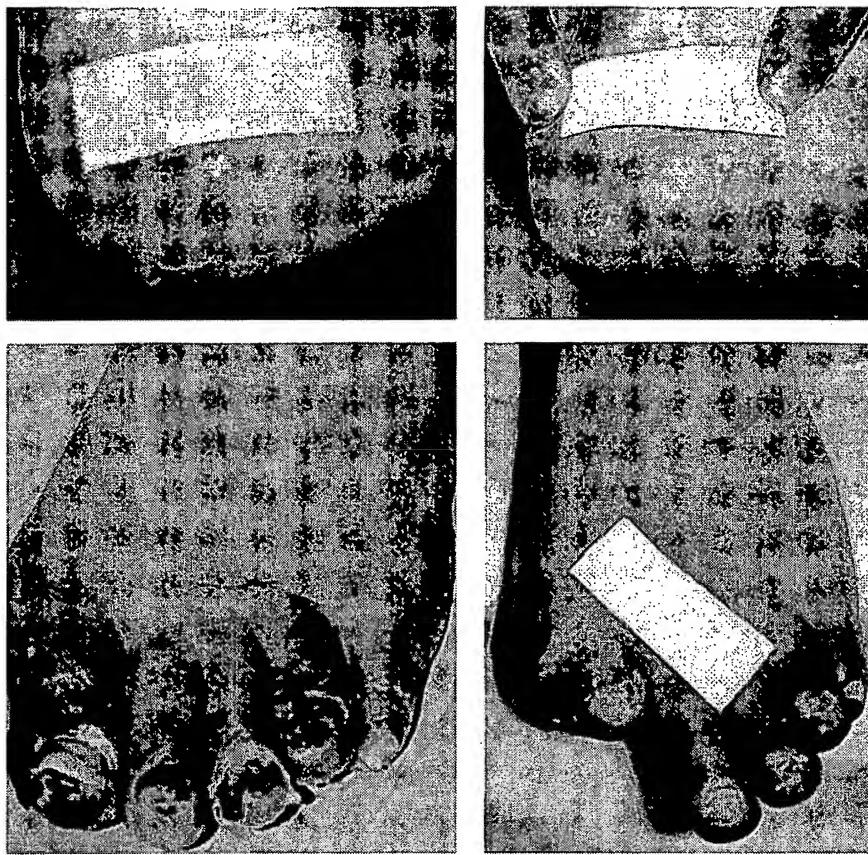


present study may have failed to depict the full extent of angiogenesis achieved after phVEGF<sub>165</sub> transfection, particularly given that most newly visible collaterals were diminutive (200 to 800  $\mu\text{m}$ ).

That angiogenesis was in fact therapeutic in the present investigation was shown by concomitant reduction in rest pain and/or a favorable impact on limb integrity. Rest pain resolved in all 3 of the patients who presented with rest pain alone. Ischemic ulcers present in 7 limbs healed or improved markedly in 4 patients; this included 3 patients recommended for below-knee amputation in whom successful limb salvage was achieved. Given the poor prognosis for patients with chronic critical limb ischemia, in whom the possibility of spontaneous improvement is remote,<sup>12</sup> the outcome in this initial cohort is thus encouraging.

Beginning with the reports of Wolff et al,<sup>39</sup> work from several laboratories<sup>40-48</sup> convincingly demonstrated evidence of transgene expression after direct injection of nonviral, covalently closed pDNA into skeletal muscle. The conceptual basis for therapeutic angiogenesis after phVEGF<sub>165</sub> gene transfer in particular has been established previously by our laboratory.<sup>49,50</sup> The results of the present trial extend previous findings from studies performed in live animals<sup>46</sup> to patients with advanced peripheral artery disease.

The failure of previous gene therapy trials to yield evidence of clinical success has been attributed to gene delivery,



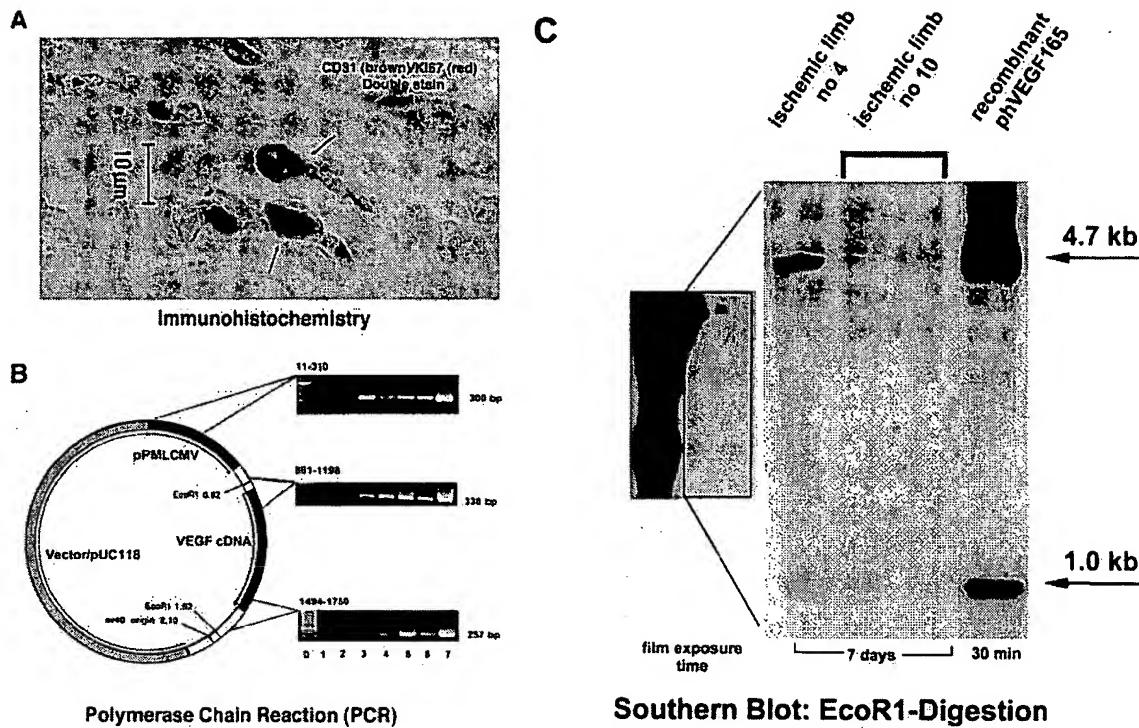
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**Figure 5.** Limb salvage after gene therapy in a 39-year-old woman (patient 8 and 4, Table). This patient presented with a 3-month history of gangrene of distal one-half of right foot. Although ABI in right limb improved by 0.27, forefoot gangrene was not reversed, and she underwent below-knee amputation. While she was being treated for right limb, however, she developed gangrene of left great toe and shortly after, of 4 remaining left toes as well. After gene transfer to left limb, her ABI increased by 0.22 in association with angiographically demonstrable new collateral vessels. Although she required a great toe amputation, operative site healed promptly, and remaining 4 toes recovered completely, including restoration of normal nail growth.

specifically the inability to deliver genes efficiently and to obtain sustained expression.<sup>51</sup> Those cases in which phVEGF<sub>165</sub> gene therapy led to successful clinical outcomes in this clinical trial suggest that the success of gene therapy is not solely a function of transfection efficiency, nor is it necessarily dependent on protracted gene expression. Several aspects of the gene, protein, and target tissue may have contributed to successful modulation of the host phenotype, despite the relatively low transfection efficiency typically associated with naked DNA. First, VEGF, as noted above, is actively secreted by intact cells; previous studies in our laboratory<sup>52</sup> have documented that genes that encode for secreted proteins, as opposed to proteins that remain intracellular, may yield meaningful biological outcomes because of paracrine effects of the secreted gene product. Second, heparin avidity of the VEGF<sub>165</sub> isoform promotes binding to cell surface and matrix heparan sulfates that may create a biological reservoir of the secreted protein, enhancing the temporal opportunity for bioactivity. Third, although endothelial cells were previously viewed solely as the target for VEGF, it is now clear that endothelial cells subjected to hypoxia can synthesize VEGF as well.<sup>53</sup> This autocrine feature of VEGF creates the opportunity for amplifying the effects of even a small amount of exogenous VEGF, because endothelial cell proliferation in the ischemic territory creates additional potential cellular sources of VEGF synthesis and secretion. Fourth, VEGF inhibits apoptosis,<sup>54</sup> in part by upregulating endothelial cell expression of fibronectin and  $\alpha_v\beta_3$ ,<sup>54,55</sup> thus preserving the survival signal generated by attachment of endothelial cells to their extracellular matrix.

Such reduction in endothelial cell apoptosis would be expected to complement the mitogenic effect of VEGF, resulting in a further net increase in endothelial cell viability. Fifth, with regard to the target of gene therapy, it has been noted<sup>14,26,49</sup> that VEGF-induced angiogenesis is not indiscriminate or widespread but rather is restricted to sites of ischemia. This appears to result from paracrine upregulation of the principal high-affinity VEGF receptor (*Kdr*) in response to factors released from hypoxic skeletal myocytes.<sup>56</sup> Receptor upregulation on endothelial cells within the region of lower-limb or myocardial ischemia thus enables these cells to act as magnets for any VEGF secreted into the ischemic milieu. Finally, the fact that the host tissues are by definition hypoxic may directly aid intramuscular transfer of naked DNA, because transfection efficiency is augmented when the injected skeletal muscle is ischemic.<sup>40,46</sup>

Previous work from our laboratory established that phVEGF<sub>165</sub> transgene expression is limited to <30 days in animal models of limb ischemia.<sup>26,46,49</sup> Although Southern blot and PCR analyses indicated that small amounts of plasmid DNA were preserved in tissue specimens derived from 2 amputees in this clinical trial, we have no evidence to suggest that transgene expression is more protracted in human subjects than in our animal models. Fortunately, however, it appears that in both animals and humans, collateral vessel development sufficient to restore limb perfusion to satisfactory resting levels occurs within this time interval. Cessation of gene expression beyond this time point can be considered to constitute an inherent safety feature of phVEGF<sub>165</sub> gene



### Southern Blot: EcoR1-Digestion

**Figure 6.** Immunohistochemical and molecular analyses of tissue specimens derived from 2 amputees 8 and 10 weeks after gene therapy (limbs 4 and 10, Table). A, Double immunohistochemical staining of tissue specimen with monoclonal antibody to CD31 (brown) and polyclonal antibody to Ki-67 (red) shows proliferating microvascular endothelial cells (arrows). B, PCR demonstrates amplification of phVEGF<sub>165</sub> DNA fragments in skin and skeletal muscle specimens. Lane 0 shows 100-bp ladder; lane 2, reaction mixture without tissue DNA; lane 3, negative control (skin specimen from untreated patient); lanes 4 to 6, specimens derived from phVEGF<sub>165</sub>-treated amputee (patient 4, Table); lane 4, skin; lane 5, gastrocnemius muscle (remote from site of transfection); lane 6, tibialis anterior muscle (transfection site); lane 7, positive control (purified phVEGF<sub>165</sub>). Amplified fragments had predicted sizes of 300, 338, and 257 bp, spanning the CMV promoter/enhancer region (5'-CCCGACATTGATTATGA-3' and 5'-CGGGCCATTACCGTCAT-3'; position 11 to 300), proximal VEGF encoding region and junction between VEGF encoding region and promoter (5'-GCCTTCTCTCCACAGGT-3' and 5'-GTACTCGATCTCATCAGG-3'; position 861 to 1198), and distal VEGF encoding region and junction between VEGF encoding region and SV40 polyadenylation sequence (5'-CGCGTTGCAAGGCGAGGC-3' and 5'-GGACCCAAAGTGCTCTGC-3'; position 1494 to 1750), respectively. C, Southern blot analysis of EcoR1-digested total cellular DNA (30  $\mu$ g) (lane 1, patient 4, Table; lanes 2 and 3, patient 10, Table), and 0.5  $\mu$ g (lane 4) of purified phVEGF<sub>165</sub> DNA hybridized with two  $^{32}$ P-labeled phVEGF<sub>165</sub> specific probes (position 389 to 1068 and position 991 to 1778) showed persistence of complete plasmid DNA (EcoR1-digested 4703- and 998-bp fragments) in skeletal muscle specimens derived from 2 amputees (patients 4 and 10, Table).

transfer that protects the organism from indefinite constitutive expression of an angiogenic growth factor.

Several caveats regarding this preliminary clinical experience must be acknowledged. First, it is theoretically possible that VEGF expression resulting from gene transfer could promote the development of a tumor that is currently too small to be recognized. Previous laboratory studies, however, have established that VEGF expression, although sufficient to promote a growth process, did not lead to malignant proliferation or to metastasis, a finding in agreement with the notion that stimulation of angiogenesis is necessary but not sufficient for malignant growth.<sup>34,57</sup> It is also theoretically possible that VEGF may aggravate deteriorating eyesight due to diabetic retinopathy.<sup>33</sup> To date, however, no change in visual acuity has been observed in any patient treated with phVEGF<sub>165</sub> gene transfer. Nevertheless, these findings are preliminary and do not establish the long-term safety of VEGF, administered either as a gene or gene product. Second, although it is conceivable that continuous, predominantly local production of VEGF resulting from the transgene may be preferable, from the standpoints of both safety and efficacy, to a single larger dose of recombinant protein,

this notion remains to be proven. Preliminary clinical trials of recombinant VEGF protein therapy have confirmed that mild hypotension seen in preclinical studies<sup>15,58</sup> may be seen in humans as well (unpublished data). Presumably, the route and/or dose of recombinant protein delivery can be adjusted to address this issue. Clearly, further clinical studies of both recombinant protein and alternative dosing regimens of gene therapy will be required to define the relative merits of each approach. Third, we cannot exclude the possibility that these encouraging preliminary results might have been made more substantial and/or uniform by the use of alternative vector systems and/or dosing strategies.<sup>45,48,51,59</sup>

In summary, these preliminary data may be cautiously interpreted to support both the strategy of intramuscular gene therapy and the concept of therapeutic angiogenesis for treatment of selected patients with critical limb ischemia.

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## References

- European Working Group on Critical Leg Ischemia. Second European consensus document on chronic critical leg ischemia. *Circulation*. 1991; 84(suppl IV):IV-1-IV-26.
- Dormandy J, Mahir M, Ascady G, Balsano F, De Leeuw P, Blombery P, Bousser MG, Clement D, Coffman J, Deutschnoff A, Bletry O, Hampton J, Hahler F, Ohlin P, Rieger H, Strandén E, Turpie AGG, Urai L, Verstraete M. Fate of the patient with chronic leg ischaemia. *J Cardiovasc Surg*. 1989;1:50-57.
- Rutherford RB, Flanigan DP, Gupta SK, Johnston KW, Karmondy A, Whittemore AD, Baker D, Ernst CB, Jamieson C, Mehta S. Suggested standards for reports dealing with lower extremity ischemia. Ad Hoc Committee on Reporting Standards, Society for Vascular Surgery/North American Chapter, International Society for Cardiovascular Surgery. *J Vasc Surg*. 1986;4:80-94.
- Albers M, Fratzei AC, DeLucia N. Assessment of quality of life of patients with severe ischemia as a result of infringuinal arterial occlusive disease. *J Vasc Surg*. 1992;16:54-59.
- Isner JM, Rosenfield K. Redefining the treatment of peripheral artery disease. *Circulation*. 1993;88:1534-1557.
- Most RS, Sinnock P. The epidemiology of lower extremity amputations in diabetic individuals. *Diabetes Care*. 1983;6:87-91.
- Taylor LM Jr, Porter JM. Natural history and non-operative treatment of chronic lower extremity ischemia. In: Rutherford RB, ed. *Vascular Surgery*. Philadelphia, Pa: WB Saunders; 1989:656.
- Wolfe JHN. Defining the outcome of critical ischemia: a one year prospective study. *Br J Surg*. 1986;73:321-328.
- Eneroth M, Persson BM. Amputation for occlusive arterial disease: a multicenter study of 177 amputees. *Int Orthop*. 1992;16:382-387.
- Campbell WB, Johnston JA, Kernick VF, Rutter EA. Lower limb amputation: striking the balance. *Ann R Coll Surg Engl*. 1994;76:205-209.
- Dawson I, Keller BP, Brand R, Pesch-Batenburg J, Hajo van Bockel J. Late outcomes of limb loss after failed infringuinal bypass. *J Vasc Surg*. 1995;21:613-622.
- Skinner JA, Cohen AT. Amputation for premature peripheral atherosclerosis: do young patients do better? *Lancet*. 1996;348:1396.
- Baffour R, Berman J, Garb JL, Rhee SW, Kaufman J, Friedmann P. Enhanced angiogenesis and growth of collaterals by in vivo administration of recombinant basic fibroblast growth factor in a rabbit model of acute lower limb ischemia: dose-response effect of basic fibroblast growth factor. *J Vasc Surg*. 1992;16:181-191.
- Takeshita S, Zheng LP, Brogi E, Kearney M, Pu LQ, Bunting S, Ferrara N, Symes JF, Isner JM. Therapeutic angiogenesis: a single intra-arterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hindlimb model. *J Clin Invest*. 1994;93:662-670.
- Hariawala M, Horowitz JR, Esakof D, Sheriff DD, Walter DH, Chaudhry GM, Desai V, Keyt B, Isner JM, Symes JF. VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. *J Surg Res*. 1996;63:77-82.
- Banai S, Jaklitsch MT, Shou M, Lazarous DF, Scheinowitz M, Biro S, Epstein SE, Unger EF. Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation*. 1994;89:2183-2189.
- Pearlman JD, Hibberd MG, Chuang ML, Harada K, Lopez JJ, Gladston SR, Friedman M, Sellke FW, Simons M. Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. *Nat Med*. 1995;1:1085-1089.
- Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*. 1983;219:983-985.
- Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*. 1989;246:1306-1309.
- Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT. Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science*. 1989;246:1309-1312.
- Plouet J, Schilling J, Gospodarowicz D. Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. *EMBO J*. 1989;8:3801-3806.
- Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun*. 1989;161:851-855.
- D'Amore PA, Thompson RW. Mechanisms of angiogenesis. *Annu Rev Physiol*. 1987;49:453-464.
- Folkman J, Shing Y. Angiogenesis. *J Biol Chem*. 1992;267:10931-10934.
- Risau W. Mechanisms of angiogenesis. *Nature*. 1997;386:671-674.
- Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Razvi S, Walsh K, Symes J. Clinical evidence of angiogenesis following arterial gene transfer of phVEGF<sub>165</sub>. *Lancet*. 1996;348:370-374.
- Standards of Practice Committee of the Society of Cardiovascular and Interventional Radiology. Guidelines for percutaneous transluminal angioplasty. *J Vasc Interv Radiol*. 1990;1:5-13.
- Rutherford RB, Becker GJ. Standards for evaluating and reporting the results of surgical and percutaneous therapy for peripheral arterial disease. *Radiology*. 1991;181:277-281.
- Tischer E, Mitchell R, Hartmann T, Silva M, Gospodarowicz D, Fiddes J, Abraham J. The human gene for vascular endothelial growth factor: multiple protein forms are encoded through alternative exon splicing. *J Biol Chem*. 1991;266:11947-11954.
- Summer DS, Thiele BI. The vascular laboratory. In: Rutherford RB, ed. *Vascular Surgery*. Philadelphia, Pa: WB Saunders; 1995:45-64.
- Owen RS, Carpenter JP, Baum RA, Perloff LJ, Cope C. Magnetic resonance imaging of angiographically occult runoff vessels in peripheral arterial occlusive disease. *N Engl J Med*. 1992;326:1577-1581.
- Couffinhal T, Kearney M, Witzenbichler B, Chen D, Murohara T, Losordo DW, Symes JF, Isner JM. Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) in normal and atherosclerotic human arteries. *Am J Pathol*. 1997;150:1673-1685.
- Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Theme H, Iwamoto MA, Parke JE, Nguyen MD, Aiello LM, Ferrara N, King GL. Vascular endothelial growth factor in ocular fluids of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med*. 1994;331:1480-1487.
- Ferrara N, Winer J, Burton T, Rowland A, Siegel M, Phillips HS, Terrell T, Keller GA, Levinson AD. Expression of vascular endothelial growth factor does not promote transformation but confers a growth advantage in vivo to Chinese hamster ovary cells. *J Clin Invest*. 1992;91:160-170.
- Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol*. 1995;146:1029-1039.
- Hiatt WR, Hirsch AT, Regensteiner JG, Brass EP, and the Vascular Clinical Trialists. Clinical trials for claudication assessment of exercise performance, functional status, and clinical end points. *Circulation*. 1995; 92:614-621.
- Schapler W, Brahander MD, Lewi P. DNA synthesis and mitoses in coronary collateral vessels of the dog. *Circ Res*. 1971;28:671-679.
- Takeshita S, Isshiki T, Tanaka E, Eto K, Miyazawa Y, Tanaka A, Shinohara Y, Hyodo K, Ando M, Kubota M, Tanioka K, Umetani K, Ochiai M, Sato T, Mori H, Miyashita H. Use of synchrotron radiation microangiography to assess development of small collateral arteries in a rat model of hindlimb ischemia. *Circulation*. 1997;95:805-808.
- Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL. Direct gene transfer into mouse muscle in vivo. *Science*. 1990;247: 1465-1468.
- Takeshita S, Isshiki T, Sato T. Increased expression of direct gene transfer into skeletal muscles observed after acute ischemic injury in rats. *Lab Invest*. 1996;74:1061-1065.
- Stratford-Perricaudet LD, Makeh I, Perricaudet M, Briand P. Widespread long-term gene transfer to mouse skeletal muscles and heart. *J Clin Invest*. 1992;90:626-630.
- Manthorpe M, Cornefert-Jensen F, Hartikka J, Felgner J, Rundell A, Margalith M, Dwarki V. Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. *Hum Gene Ther*. 1993;4:419-431.
- Davis HL, Whalen RG, Demeneix BA. Direct gene transfer into skeletal muscle in vivo. *Hum Gene Ther*. 1993;4:151-159.
- Vitadello M, Schiavino M, Picard A, Scarpa M, Schiavino S. Gene transfer in regenerating muscle. *Hum Gene Ther*. 1994;5:11-18.
- Levy MY, Barron LG, Meyer KB, Szoka FC. Characterization of plasmid DNA transfer into mouse skeletal muscle: evaluation of uptake mechanism, expression and secretion of gene products into blood. *Gene Ther*. 1996;3:201-211.

46. Tsurumi Y, Takeshita S, Chen D, Kearney M, Rossow ST, Passeri J, Horowitz JR, Symes JF. Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. *Circulation*. 1996;94:3281-3290.
47. Tripathy SK, Svensson EC, Black HB, Goldwasser E, Margalith M, Hobart PM, Leiden JM. Long-term expression of erythropoietin in the systemic circulation of mice after intramuscular injection of a plasmid DNA vector. *Proc Natl Acad Sci USA*. 1996;93:10876-10880.
48. Hartikka J, Sawdey M, Cornefert-Jensen F, Margalith M, Barnhart K, Nolasco M, Vahlsgaard L, Meek J, Marquet M, Hobart P, Norman J, Manthorpe M. An improved plasmid DNA expression vector for direct injection into skeletal muscle. *Hum Gene Ther*. 1996;7:1205-1217.
49. Takeshita S, Tsurumi Y, Couffinhal T, Asahara T, Bauters C, Symes JF, Ferrara N, Isner JM. Gene transfer of naked DNA encoding for three isoforms of vascular endothelial growth factor stimulates collateral development in vivo. *Lab Invest*. 1996;75:487-502.
50. Takeshita S, Weir L, Chen D, Zheng LP, Riessen R, Bauters C, Symes JF, Ferrara N, Isner JM. Therapeutic angiogenesis following arterial gene transfer of vascular endothelial growth factor in a rabbit model of hindlimb ischemia. *Biochem Biophys Res Commun*. 1996;227:628-635.
51. Verma IM, Somia N. Gene therapy: promises, problems, and prospects. *Nature*. 1997;389:239-242.
52. Losordo DW, Pickering JG, Takeshita S, Leclerc G, Gal D, Weir L, Kearney M, Jekanowski J, Isner JM. Use of the rabbit ear artery to serially assess foreign protein secretion after site specific arterial gene transfer in vivo: evidence that anatomic identification of successful gene transfer may underestimate the potential magnitude of transgene expression. *Circulation*. 1994;89:785-792.
53. Namiki A, Brogi E, Kearney M, Wu T, Couffinhal T, Varticovski L, Isner JM. Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. *J Biol Chem*. 1995;270:31189-31195.
54. Spyridopoulos I, Brogi E, Kearney M, Sullivan AB, Cetruolo C, Isner JM, Losordo DW. Vascular endothelial growth factor inhibits endothelial cell apoptosis induced by tumor necrosis factor-alpha: balance between growth and death signals. *J Mol Cell Cardiol*. 1997;29:1321-1330.
55. Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Perruzzi CA, Detmar M. Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the avb3 integrin, osteopontin, and thrombin. *Am J Pathol*. 1996;149:293-305.
56. Brogi E, Schatterman G, Wu T, Kim EA, Varticovski L, Keyt B, Isner JM. Hypoxia-induced paracrine regulation of VEGF receptor expression. *J Clin Invest*. 1996;97:469-476.
57. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971;285:1182-1186.
58. Horowitz JR, Rivard A, van der Zee R, Hariawala MD, Sheriff DD, Esakof DD, Chaudhry M, Symes JF, Isner JM. Vascular endothelial growth factor/vascular permeability factor produces nitric oxide-dependent hypotension. *Arterioscler Thromb Vasc Biol*. 1997;17:2793-2800.
59. Leiden JM. Gene therapy: promise, pitfalls, and prognosis. *N Engl J Med*. 1995;333:871-873.